Research Article

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A CCRK-EZH2 epigenetic circuitry drives hepatocarcinogenesis and associates with tumor recurrence and poor survival of patients

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Background & Aims: Aberrant chromatin modification is a key feature of hepatocellular carcinoma (HCC), which is characterized by strong sexual dimorphism. Both enhancer of zeste homolog 2 (EZH2) and cell cycle-related kinase (CCRK) contribute to hepatocarcinogenesis, yet whether the two oncogenic factors have functional crosstalk is unknown.

Methods: Cellular proliferation and tumorigenicity upon transgenic expression and RNA interference were determined by colony formation and soft agar assays, xenograft, orthotopic and diethylnitrosamine-induced HCC models. Gene regulation was assessed by chromatin immunoprecipitation, site-directed mutagenesis, luciferase reporter, co-immunoprecipitation and expression analyses. Protein levels in clinical specimens were correlated with clinicopathological parameters and patient survival rates.

Results: Ectopic CCRK expression in immortalized human liver cells increased EZH2 and histone H3 lysine 27 trimethylation (H3K27me3) to stimulate proliferation and tumor formation. Conversely, knockdown of CCRK reduced EZH2/H3K27me3 levels and decreased HCC cell growth, which could be rescued by EZH2 over-expression. Mechanistically, GSK-3β phosphorylation by CCRK activated a β-catenin/TCF/E2F1/EZH2 transcriptional feedback loop to epigenetically enhance androgen receptor (AR) signaling. Simultaneously, the phosphorylation of AKT/EZH2 by CCRK facilitated the co-occupancy of *CCRK* promoter by EZH2-AR and its subsequent transcriptional activation, thus forming a self-reinforcing circuitry. Lentiviral-mediated knockdown of *CCRK*, which abrogated the phosphorylation-transcriptional network, prevented diethylnitrosamine-induced tumorigenicity. More importantly, the hyperactivation of the CCRK-EZH2 circuitry in human HCCs correlated with tumor recurrence and poor survival.

Conclusions: These findings uncover an epigenetic vicious cycle in hepatocarcinogenesis that operates through reciprocal regulation of CCRK and EZH2, providing novel therapeutic strategy for HCC.

Keywords: Androgen receptor; Chromatin modifications; Hepatocellular carcinoma; Gender disparity; Kinase.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; E2F1, E2F transcription factor 1; EZH2, enhancer of zeste homolog 2; PRC2, polycomb repressive complex 2; H3K27me3, trimethylation of lysine 27 at histone H3; AR, androgen receptor; CCRK, cell cycle-related kinase; GSK-3β, glycogen synthase kinase 3β; TCF, T-cell factor; DEN, diethylnitrosamine; RT, reverse transcription; ChIP, chromatin immunoprecipitation; shRNA, short-hairpin RNA; KD, kinase-defective; WT, wild-type; p-GSK3β^{Ser9}, GSK-3β phosphorylation at serine9; dn, dominant-negative; siRNA, small-interfering RNA; dp, dominant-positive; p-AR^{Ser81}, AR phosphorylation at serine81; p-AKT^{Ser473}, AKT phosphorylation at serine473; p-EZH2^{Ser21}, EZH2 phosphorylation at serine21; ARE, androgen-responsive element.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy worldwide with a strong sexual dimorphism. Male to female ratio averages between 2:1 and 7:1 in HCC associated with viral infection and non-alcoholic fatty liver diseases [1,2]. Hepatitis B virus (HBV) infection accounts for about 60% of the total liver cancer in developing countries. Unlike most other cancers, the incidence and mortality of HCC have increased in Western countries in the past decade due to hepatitis C virus infection and the obesity epidemic. Although the multikinase inhibitor Sorafenib can improve survival of HCC patients, the durability of treatment response is still far from satisfactory [3]. The alarmingly high failure rate of phase III molecular therapy trials in the past 5 years further underscores the compelling need for novel drug targets [4].

HCC pathogenesis is a complex process driven by accumulating genetic and epigenetic alterations [5,6]. Integrative oncogenomic analysis has uncovered prominent oncogenes such as *CTNNB1*, *AKT*, and *E2F transcription factor 1* (*E2F1*) in HCC [7]. While these oncogenic pathways are often concordantly activated in molecular subtypes of human HCCs [8,9], the mechanistic basis of the signaling connections is largely unknown.

Delineation of the signaling hubs that contribute to hepatocarcinogenesis may greatly advance therapeutic development [3,10].

One of the most remarkable discoveries in cancer genomics is the recurrent somatic mutations of epigenetic-modifying genes [11,12]. Although each HCC case has a unique mutational profile, up to 50% of cases are estimated to harbor mutations in different chromatin regulators [4,10,13]. Enhancer of zeste homolog 2 (EZH2), a subunit of Polycomb repressive complex 2 (PRC2) that catalyzes the repressive histone H3 lysine 27 trimethylation (H3K27me3) [14], is subject to genetic alteration in hematological malignancies and over-expression in a wide range of solid tumors including HCC [12,15]. Numerous evidence have suggested that aberrations of EZH2 represent early events that render cells susceptible to oncogenic transformation and refractory to differentiation program [15,16]. However, the mechanisms governing the over-expression and functions of this master chromatin regulator in HCC remain elusive.

Concordant with the male predominance of HCC, the sex hormone receptor androgen receptor (AR) plays a dominant role in hepatocarcinogenesis [17]. Our prior work underpinned cell cycle-related kinase (CCRK, also known as cyclin-dependent kinase 20) as an oncogenic effector of AR in HCC [18,19]. Despite its strong oncogenicity, no study has explored the epigenetic regulation by CCRK. Here we show that CCRK induces EZH2 up-regulation and phosphorylation in an epigenetic circuitry consisting of glycogen synthase kinase 3β (GSK-3β), β-catenin, T-cell factor (TCF), E2F1, AKT, and AR. This signaling network is activated during diethylnitrosamine (DEN)-induced hepatic tumorigenicity which can be dramatically suppressed by lentiviral-mediated knockdown of *CCRK*. As the self-reinforcing circuit hyperactivates in human hepatocarcinogenesis and associates with poor prognosis of patients, our data raise the possibility that CCRK is a promising molecular target for therapeutic intervention.

Materials and methods

Cell culture, transfection and functional assays

LO2, HuH7, PLC5, and SK-Hep1 cells were cultured in DMEM supplemented with 10% FBS (Hyclone). Cell transfection was conducted using X-tremeGene Transfection Reagent (Roche) or HiPerfect (Qiagen) according to the manufacturer's instructions. The information of small-interfering RNA (siRNA), short-hairpin RNA (shRNA) constructs, expression vectors, and luciferase reporter constructs are described in the Supplementary material. Anchorage-dependent and independent growth were analyzed by colony formation and soft agar assays, respectively, as previously described [18].

Quantitative RT-PCR, ChIP-PCR, immunoblotting, co-immunoprecipitation and immunohistochemistry

cDNA synthesis from RNA purified from cell lines and mouse samples was conducted using reverse transcription (RT) Master Kit (Invitrogen). Chromatin immunoprecipitation (ChIP) assays were performed as previously described [18,20]. Detailed protocols of quantitative RT-PCR, ChIP-PCR, cell lysis, immunoblotting and co-immunoprecipitation are described in the Supplementary material. Cell proliferation in mouse tissues was assayed by Ki67 immunohistochemistry as previously described [19].

In vivo tumorigenicity assays

The xenograft and orthotopic models were performed using athymic nude mice (n = 7 per group) as previously described [18]. The CCRK knockdown experiment in the DEN (2 mg/kg; Sigma-Aldrich) model was performed using male C57BL/6 mice (n = 12 per group) as previously described [19]. Lentiviruses encoding shRNA against *CCRK* or a control sequence were packaged according to the manufacturer's instructions (Dharmacon). All animal studies were reviewed and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Patients samples

Patients who underwent hepatectomy for HCC at the Prince of Wales Hospital (Hong Kong) were included in this study. All patients gave written informed consent on the use of clinical specimens for research purposes. Studies using human tissue were reviewed and approved by the Joint CUHK-NTEC Clinical Research Ethics Committee.

Statistical analysis

Unless otherwise indicated, data are presented as mean ± standard deviation of three independent experiments. Statistical analysis is described in the Supplementary material. A two tailed p value of less than 0.05 was considered statistically significant.

Results

EZH2 is required for CCRK-induced hepatocellular proliferation and transformation

To investigate the role and function of EZH2 in CCRK-induced hepatocarcinogenesis, we modulated gene expressions and performed colony formation and soft agar assays using both ectopic expression and knockdown approaches. Ectopic *CCRK* expression induced EZH2 and global H3K27me3 levels (Fig. 1A, left) and markedly increased focus formation (Fig. 1A, right) and anchorage-independent growth (Fig. 1B) of LO2 cells, an immortalized human liver cell line [18]. Notably, silencing of EZH2 by shRNA abrogated the induced proliferation and malignant transformation in CCRK-expressing cells (Fig. 1A–B). Conversely, shRNA-mediated knockdown of *CCRK* in PLC5 HCC cells dramatically reduced EZH2 and H3K27me3 levels (Fig. 1C, left) and decreased anchorage-dependent (Fig. 1C, right) and -independent growth (Fig. 1D), which could be rescued by ectopic *EZH2* expression (Fig. 1C–D). Consistently, downregulation of EZH2 in PLC5 and HuH7 HCC cells also resulted in significant growth inhibition (Supplementary Fig. 1A–B). We next performed *in vivo* experiments to validate the functional relationship between CCRK and EZH2. *CCRK* stably-transfected LO2 cells (CCRK-shCtrl-LO2) displayed significant increase in tumor volume and weight in the xenograft model when compared with empty vector-transfected cells (Vec-shCtrl-LO2), whereas knockdown of EZH2 completely attenuated the tumorigenicity of CCRK-shEZH2-LO2 cells (Fig. 1E). Concordantly, in an orthotopic model where the stable cell xenografts were implanted into the recipient livers, CCRK-induced intrahepatic tumorigenicity was abolished by EZH2 knockdown (Fig. 1F). These results demonstrate that EZH2 upregulation is required for CCRK-induced hepatocarcinogenesis.

CCRK activates a GSK-3β/β-catenin/TCF/E2F1 cascade to upregulate EZH2

We have recently identified GSK-3β as a CCRK kinase target which mediates β-catenin activation in liver and HCC cells [18,19]. To elucidate whether the GSK-3β/β-catenin signaling is involved in CCRK-induced EZH2 upregulation, we first employed a kinase-defective (KD) *CCRK* mutant whose Thr161 active site was replaced by alanine (T161A). Ectopic expression of wild-type

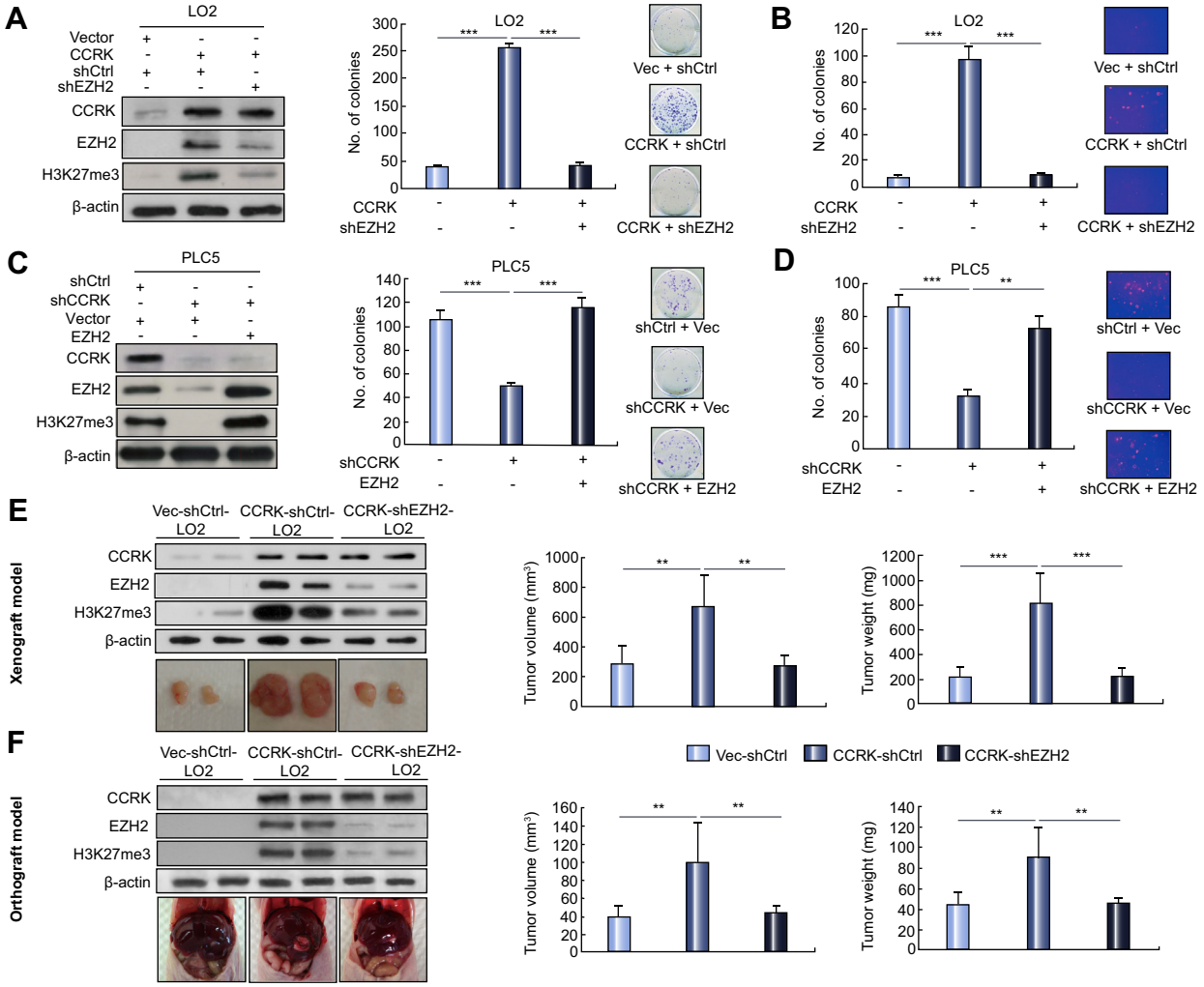
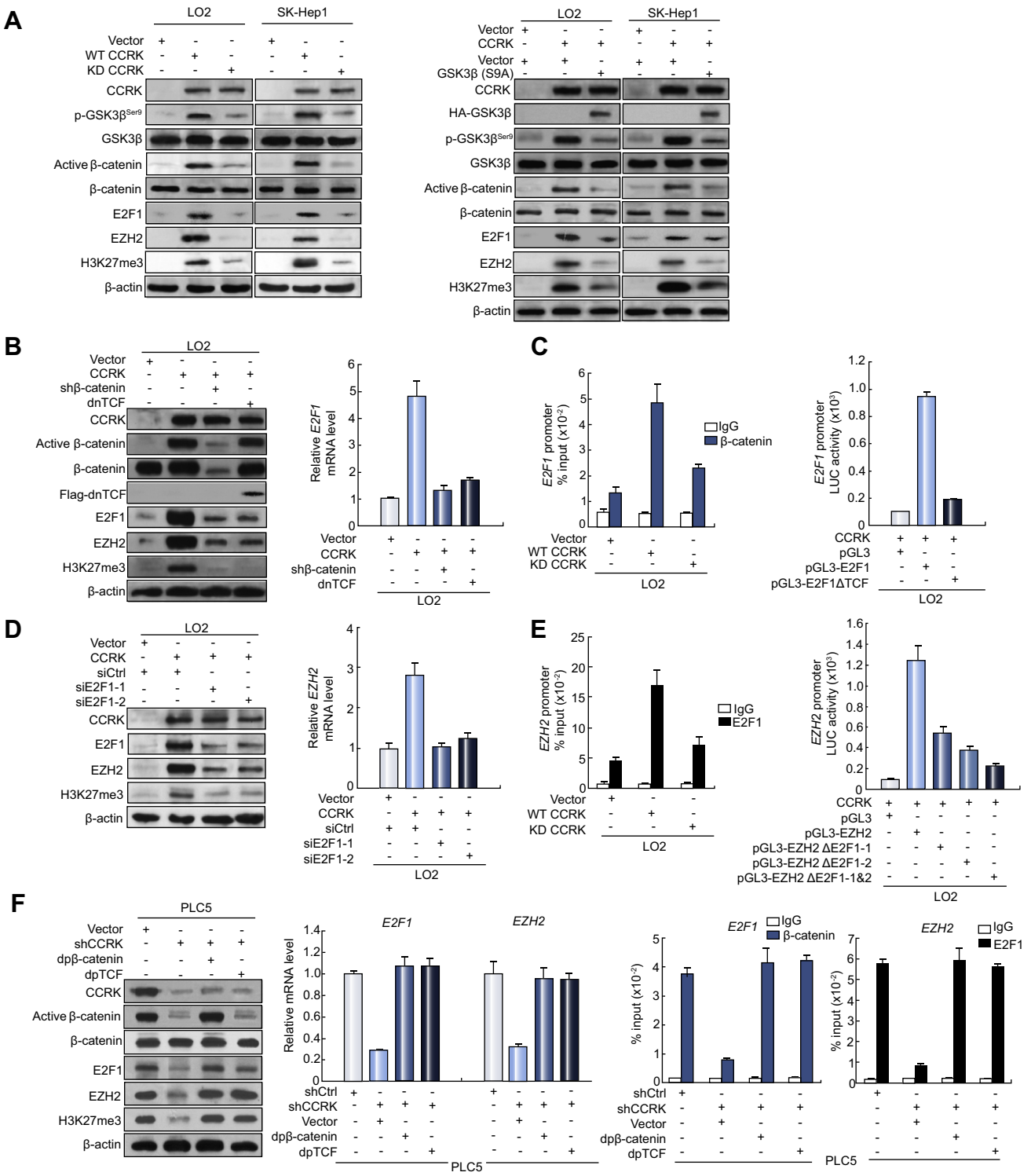


Fig. 1. EZH2 is required for CCRK-induced hepatocellular proliferation and transformation. (A) Immunoblot analysis of CCRK, EZH2, and H3K27me3 expression (left), focus formation (right), and (B) anchorage-independent growth in control and CCRK-expressing LO2 cells with or without EZH2 knockdown. (C and D) Same experiments for control and CCRK-silencing PLC5 cells with or without EZH2 over-expression. (E) Tumorigenicity of control and CCRK-expressing LO2 cells with or without EZH2 knockdown were determined by xenograft and (F) orthotopic mouse models (n = 7 per group). Immunoblot analysis of CCRK, EZH2, H3K27me3 expression and representative images of the tumors in each group are shown. Tumor volume and weight of the excised tumors in the three groups were measured. $^{**}p$ <0.01; $^{***}p$ <0.001. (This figure appears in color on the web.)

(WT) but not KD CCRK induced an upregulation in EZH2 and H3K27me3 levels in both LO2 liver and SK-Hep1 HCC cells, which was associated with increased GSK-3β phosphorylation at serine9 (p-GSK3β^{Ser9}) and active β-catenin level (Fig. 2A, left). Moreover, inhibition of p-GSK3β^{Ser9} by a dominant-inhibitory S9A-GSK-3β mutant impaired not only CCRK-induced β-catenin activation, but also EZH2 and H3K27me3 (Fig. 2A, right). To determine the role of β-catenin signaling, we ablated β-catenin by shRNA or expressed dominant-negative TCF (dnTCF) in CCRK-expressing LO2 cells. Blockage of β-catenin/TCF signaling dramatically reduced CCRK-induced EZH2 and H3K27me3 levels (Fig. 2B). Moreover, CCRK increased the expression of *E2F1*, an EZH2 trans-activator [21] in a GSK-3β/β-catenin/TCF-dependent manner (Fig. 2A–B). WT but not KD CCRK induced β-catenin occupancy (Fig. 2C, left) to drive *E2F1* transcription, as deletion of TCF binding site rendered the *E2F1* promoter non-responsive to CCRK induction (Fig. 2C, right). Further, knockdown of E2F1 by two independent siRNAs abrogated CCRK-induced E2F1 binding to *EZH2* promoter and EZH2/H3K27me3 expression levels (Fig. 2D; Supplementary Fig. 2A). Accordingly, WT but not KD CCRK induced E2F1 promoter binding (Fig. 2E, left) to activate *EZH2* transcription, because deletion of both E2F1 binding sites in the *EZH2* promoter abolished transcriptional activation by CCRK (Fig. 2E, right).

We further confirmed these results by two HCC cell lines, namely the CCRK-low-expressing SK-Hep1 cells and the CCRK-highly-expressing PLC5 cells using over-expression (Supplementary Fig. 3A–D) and knockdown approaches, respectively. Knockdown of *CCRK* in PLC5 cells dramatically reduced β-catenin signaling and the levels of E2F1, EZH2, and H3K27me3, which were restored by ectopic expression of dominant-positive β-catenin (dpβ-catenin) or TCF (dpTCF) (Fig. 2F, left). Moreover, both dpβ-catenin and dpTCF alleviated the shCCRK-mediated suppression of β-catenin and E2F1 binding to *E2F1* and *EZH2* promoters, respectively (Fig. 2F, right). Taken together, these findings demonstrate that CCRK phosphorylates GSK-3β to activate a β-catenin/TCF/E2F1 transcriptional cascade for EZH2 up-regulation.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Cancer

CCRK induces a β-catenin/E2F1/EZH2 transcriptional feedback loop via PRC2-mediated silencing

We next examined whether CCRK induces EZH2-mediated epigenetic silencing. Consistent with our previous finding that EZH2 effectively silences Wnt antagonists via H3K27me3 in HCC [20], quantitative ChIP-PCR showed that CCRK induced EZH2 promoter occupancy on *AXIN2*, *NKD1*, *PPP2R2B*, *PRICKLE1*, and *SFRP5* in both LO2 and SK-Hep1 cells (Fig. 3A). Moreover, CCRK induced promoter occupancy of another PRC2 component SUZ12 and the repressive H3K27me3 but not the active H3K4me3 mark (Fig. 3A). In contrast, the KD CCRK mutant, which was unable to upregulate EZH2 (Fig. 2A), failed to induce PRC2 and H3K27me3 occupancy on these promoters (Fig. 3A). In accord, quantitative RT-PCR showed that WT but not KD CCRK significantly reduced the mRNA expression of these Wnt antagonists (Fig. 3B). On the other hand, downregulation of EZH2 by shRNA in PLC5 cells reduced H3K27me3, active β-catenin and E2F1 levels (Supplementary Fig. 2B). Moreover, shEZH2 decreased β-catenin and E2F1 binding to *E2F1* and *EZH2* promoters, respectively (Supplementary Fig. 2C). These findings demonstrate that CCRK induces PRC2-mediated silencing to activate a β-catenin/E2F1/EZH2 transcriptional feedback loop.

EZH2 feedback induces CCRK expression via activation of β-catenin/AR signaling

Based on the crosstalk between AR, β-catenin and EZH2 signaling [18,20], we hypothesized that EZH2 epigenetically regulates AR signaling through β-catenin activation. Ectopic expression of WT EZH2 increased the H3K27me3 levels and expressions of AR, AR phosphorylation at serine81 (p-AR^{Ser81}) and CCRK in both LO2 and SK-Hep1 cells, indicating induction of AR transcriptional activity by EZH2 (Fig. 3C). As expected, EZH2 also increased active β-catenin level in consensus with repression of the Wnt antagonists (Fig. 3C–D). In contrast, ectopic expression of EZH2 mutant without SET domain (EZH2 ΔSET), which was unable to trimethylate H3K27, failed to repress Wnt antagonists and activate β-catenin and AR signaling (Fig. 3C–D). Notably, inhibition of β-catenin/TCF signaling by shβ-catenin or dnTCF abolished EZH2-induced AR, p-AR^{Ser81}, CCRK expressions (Fig. 3E, left) and AR-responsive transcriptional activity in LO2 cells (Fig. 3E, right). In the reciprocal experiments, ectopic expression of dpβ-catenin or dpTCF in *EZH2* knockdown PLC5 cells restored the AR transcriptional activity and CCRK upregulation (Fig. 3F). In accordance with our data above, downregulation of β-catenin in HCC cells not only reduced the AR transcriptional activity and CCRK expression but also the EZH2 and H3K27me3 levels (Supplementary Fig. 4A–B). In addition, rescue experiments have shown that AR increases EZH2 and H3K27me3 levels in a CCRK-dependent manner (Supplementary Fig. 5A–B). Overall, these data decipher a critical epigenetic component of the CCRK auto-regulatory loop.

AKT-mediated EZH2 phosphorylation is indispensable for the CCRK auto-regulatory loop

Recent studies unveiled the polycomb-independent role of EZH2 in gene activation via AKT-mediated phosphorylation [22,23]. Interestingly, we found that ectopic expression of WT but not KD CCRK induced both AKT phosphorylation at serine473 (p-AKT^{Ser473}) and EZH2 phosphorylation at serine21 (p-EZH2^{Ser21}) in LO2 and SK-Hep1 cells (Fig. 4A). Over-expression of an AKT S473A mutant, which lacks a phosphorylation site in the hydrophobic motif, abolished CCRK-induced p-EZH2^{Ser21} without affecting the total EZH2 and H3K27me3 levels (Fig. 4B). To further investigate the role of p-EZH2^{Ser21} in the AR/CCRK cascade, we expressed WT EZH2 and the S21A mutant in LO2 and SK-Hep1 cells. In contrast to the Ser21 phosphorylation-competent EZH2, the S21A mutant failed to upregulate AR and CCRK expressions (Fig. 4C, left) and the *CCRK* promoter activity (Fig. 4C, right).

p-EZH2^{Ser21}-AR interaction is required for CCRK promoter co-occupancy and transcriptional activation

We next determined how EZH2 phosphorylation regulates *CCRK* transcription. Ectopic *CCRK* expression increased AR, EZH2 and p-EZH2^{Ser21} expression (Fig. 4D, top) and induced its own transcription as shown by promoter luciferase assays in both LO2 and SK-Hep1 cells (Fig. 4D, bottom). Notably, knockdown of either AR or EZH2 in CCRK-expressing cells completely abolished CCRK auto-regulation (Fig. 4D). Deletion of the androgen-responsive element (ARE) in the *CCRK* promoter [18] prevented the transcriptional activation, indicating that the transcription was dependent on direct AR binding (Fig. 4D). Quantitative ChIP-PCR indeed confirmed that CCRK induced its own promoter occupancy by AR, EZH2 and p-EZH2^{Ser21} in both cell lines (Fig. 4E). In accordance with gene activation, CCRK increased the binding of RNA polymerase II with the active H3K4me3 but not repressive H3K27me3 mark to its promoter (Fig. 4E). In contrast, downregulation of either AR or EZH2 abrogated their promoter co-occupancy and the active chromatin state induced by CCRK (Fig. 4E).

We next conducted co-immunoprecipitation to determine whether AR physically interacts with EZH2 or p-EZH2^{Ser21} for promoter co-occupancy. WT but not KD CCRK induced a robust

Fig. 2. CCRK upregulates EZH2 expression through activation of a GSK3β/β-catenin/TCF/E2F1 cascade. (A) Immunoblot analysis of CCRK, p-GSK3β^{Ser9}, GSK-3β, active and total β-catenin, E2F1, EZH2, and H3K27me3 in LO2 and SK-Hep1 cells expressing WT and KD CCRK (left), or WT CCRK with or without S9A-GSK3β mutant compared to the vector control (right). (B) Immunoblot analysis of CCRK, active and total β-catenin, Flag-tagged dnTCF, E2F1, EZH2, and H3K27me3 and quantitative RT-PCR analysis of *E2F1* in control and CCRK-expressing LO2 cells with or without shβ-catenin and dnTCF expression. (C) Quantitative ChIP-PCR analysis of β-catenin binding on *E2F1* promoter in control, WT and KD CCRK-expressing LO2 cells (left). Enrichment was determined as a percentage of input DNA. IgG antibody was used as a negative control. The activity of the *E2F1* promoter luciferase reporter with or without TCF binding site deletion in CCRK-expressing LO2 cells (right). (D) Immunoblot and quantitative RT-PCR analyses in control and CCRK-expressing LO2 cells with or without E2F1 knockdown by two independent siRNAs. (E) Quantitative ChIP-PCR analysis of E2F1 binding on *EZH2* promoter in control, WT and KD CCRK-expressing LO2 cells (left). The activity of the *EZH2* promoter luciferase reporter with or without single or double E2F1-binding site deletion in CCRK-expressing LO2 cells (right). (F) Immunoblot and quantitative RT-PCR analyses in control and CCRK-silencing PLC5 cells with or without dpβ-catenin and dpTCF expression (left). Quantitative ChIP-PCR analysis of β-catenin and E2F1 binding on *E2F1* and *EZH2* promoters, respectively, in control and CCRK-silencing PLC5 cells with or without dpβ-catenin and dpTCF expression (right).

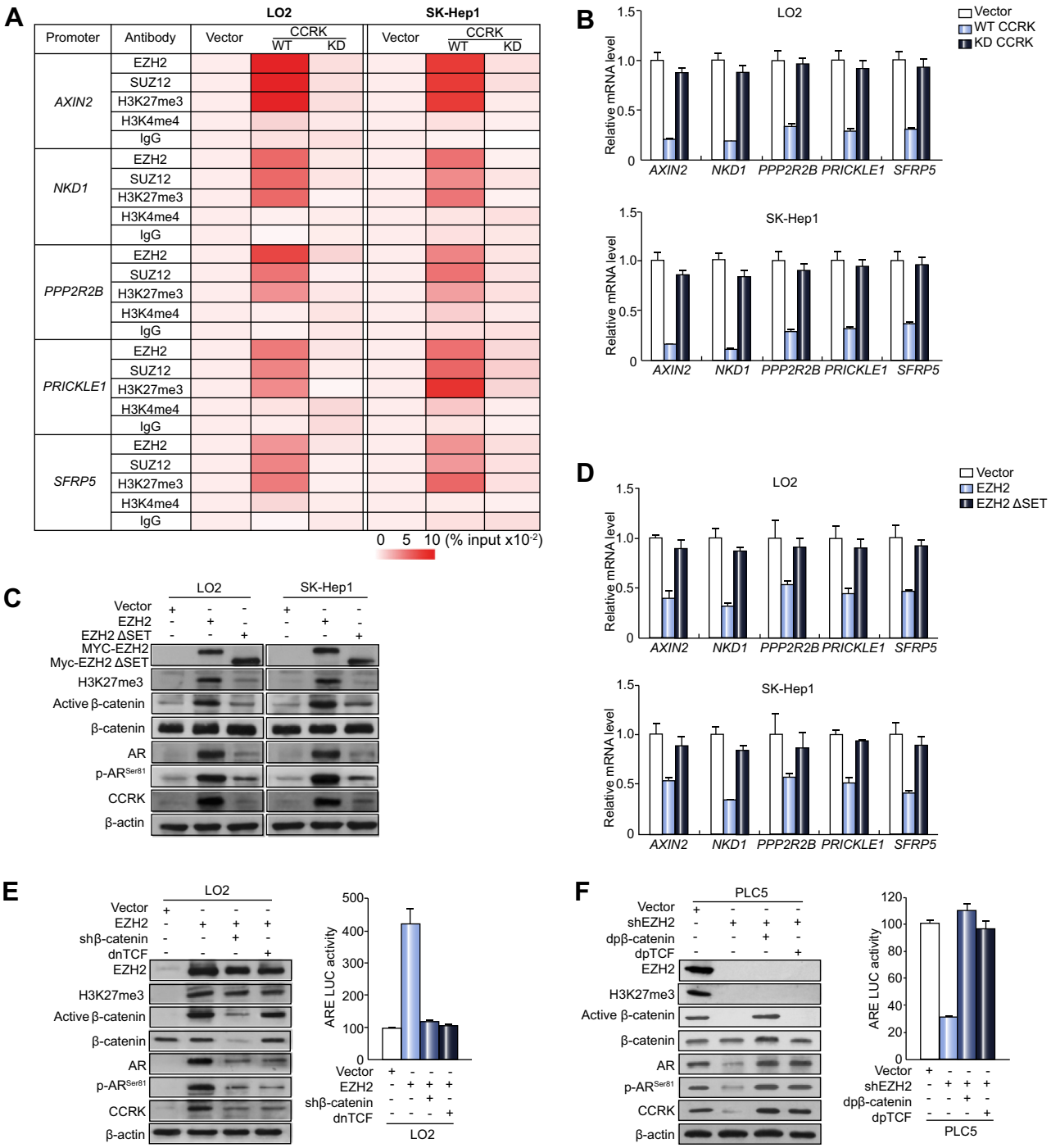

Fig. 3. CCRK induces a β-catenin-EZH2 positive feedback loop to epigenetically enhance AR signaling. (A) Heatmap showing quantitative ChIP-PCR analyses of EZH2, SUZ12, H3K27me3 and H3K4me3 occupancies on the promoters of Wnt antagonists in control, WT and KD CCRK-expressing LO2 and SK-Hep1 cells. (B) Quantitative RT-PCR analysis of the Wnt antagonists in control, WT and KD CCRK-expressing LO2 and SK-Hep1 cells. (C) Immunoblot and (D) quantitative RT-PCR analysis in control, EZH2 and EZH2 ΔSET-expressing LO2 and SK-Hep1 cells. (E) Immunoblot analysis (left) and the activity of the ARE luciferase reporter (right) in control and EZH2-expressing LO2 cells with or without shβ-catenin and dnTCF expression. (F) Same experiments for control and EZH2-silencing PLC5 cells with or without dpβ-catenin and dpTCF expression. (This figure appears in color on the web.)

physical interaction between AR and EZH2/p-EZH2^{Ser21} in LO2 and SK-Hep1 cells (Fig. 4F, left). However, co-expression of WT CCRK with either EZH2 S21A (Fig. 4F, middle) or AKT S473A mutant (Fig. 4F, right), which prohibited p-EZH2^{Ser21} (Fig. 4B), abolished the interaction even though both AR and EZH2 remained upregulated. These data demonstrate that CCRK mediated p-EZH2^{Ser21}, but not un-phosphorylated EZH2, is essential for its interaction with AR.

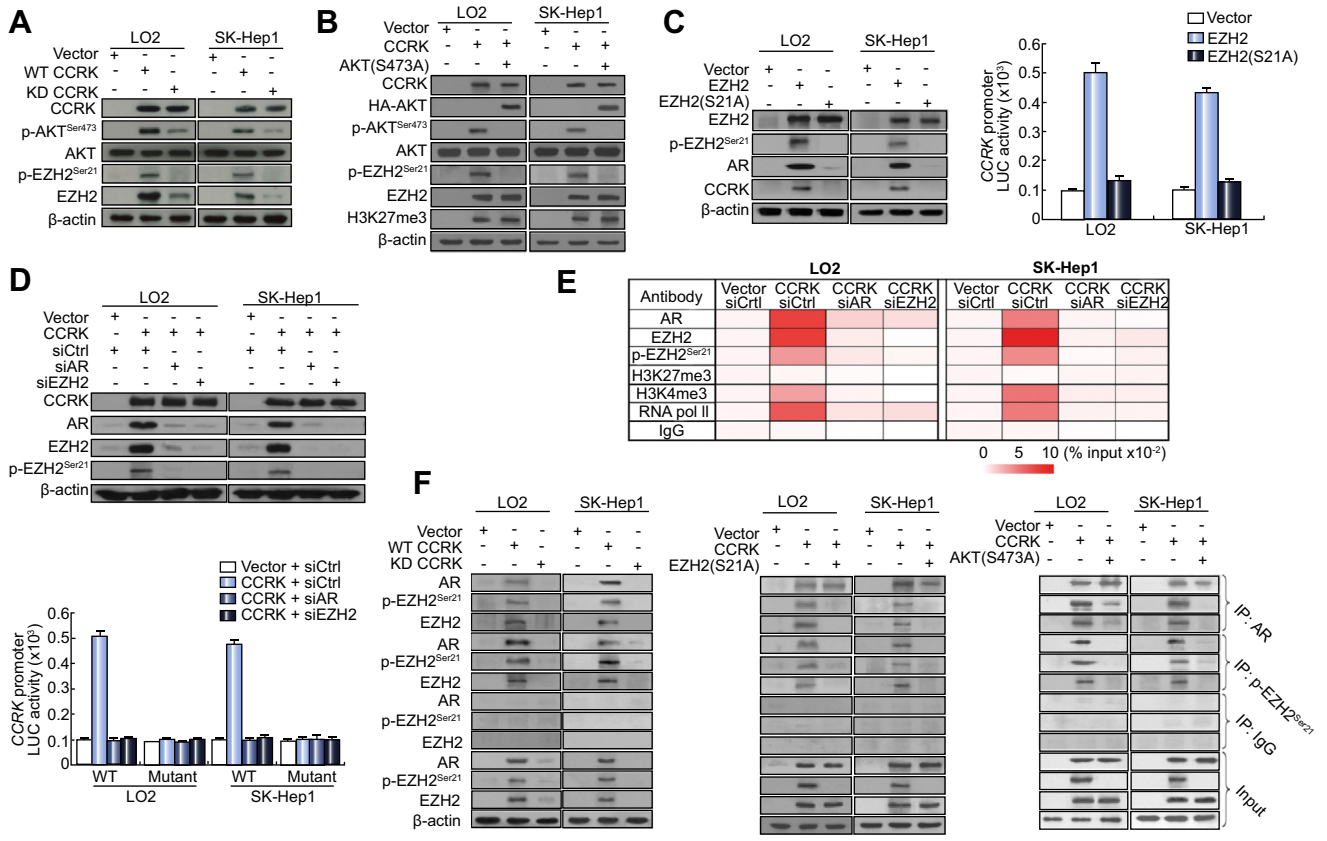

Fig. 4. CCRK-mediated EZH2 phosphorylation by AKT functions as an AR co-activator for *CCRK* promoter co-occupancy and transcriptional activation. (A) Immunoblot analysis of CCRK, p-AKT^{Ser473}, AKT, p-EZH2^{Ser21}, and EZH2 in control, WT and KD CCRK-expressing LO2 and SK-Hep1 cells. (B) Immunoblot analysis of CCRK, p-AKT^{Ser473}, AKT, p-EZH2^{Ser21}, EZH2, and H3K27me3 in control and CCRK-expressing LO2 and SK-Hep1 cells with or without S473A AKT mutant expression. (C) Immunoblot analysis (left) and the activity of *CCRK* promoter luciferase reporter (*right*) in control, WT and S21A EZH2-expressing LO2 and SK-Hep1 cells. (D) Immunoblot analysis (top), the activities of the WT *CCRK* promoter and ARE-deleted mutant luciferase reporters (bottom) and (E) the heatmap showing the quantitative ChIP-PCR analyses of AR, EZH2, p-EZH2^{Ser21}, H3K27me3, H3K4me3, and RNA polymerase II (RNA polII) occupancies on *CCRK* promoter in control and CCRK-expressing LO2 and SK-Hep1 cells with or without siRNA-mediated knockdown of AR and EZH2. (F) Co-immunoprecipitation of AR and p-EZH2^{Ser21} in control, WT and KD CCRK-expressing LO2 and SK-Hep1 cells, followed by immunoblot analysis of AR, p-EZH2^{Ser21} and EZH2 (left). Co-immunoprecipitation of AR and p-EZH2^{Ser21} in control, WT CCRK-expressing LO2 and SK-Hep1 cells with or without S21A EZH2 (middle) and S473A AKT mutant expression (right). IgG represents a control antibody used for IPs. Total lysates were used as input controls. (This figure appears in color on the web.)

The CCRK-EZH2 circuitry is activated during HCC development in mice

According to the *in vitro* data, CCRK and EZH2 form an epigenetic circuitry to stimulate hepatocellular proliferation and transformation. To validate the functional significance of the CCRK-EZH2 circuitry *in vivo*, we investigated whether it is perturbed during the development of DEN-induced hepatocarcinogenesis, a murine HCC model reminiscent of patients with poor survival [24]. At 8 months post-treatment, DEN-induced HCCs displayed aberrant expressions of AR, p-AR^{Ser81}, CCRK, p-GSK3β^{Ser9}, active β-catenin, E2F1, p-AKT^{Ser473}, p-EZH2^{Ser21}, EZH2, and H3K27me3 when compared to the adjacent non-tumoral tissues and normal livers of the untreated mice (Fig. 5A, top; Supplementary Fig. 6). In addition, the transcript levels of *Ar*, *Ccrk*, *E2f1*, and *Ezh2* were low in control liver tissues but highly elevated in tumors (Fig. 5A, bottom). In contrast, the expressions of the Wnt antagonists *Axin2*, *Nkd1*, *Ppp2r2b*, *Prickle1*, and *Sfrp5* were strongly repressed in tumors compared to control liver tissues (Fig. 5A, bottom). Notably, the aberrations of the transcript and protein levels of the CCRK-EZH2 circuit members were already evident at 6 month old DEN-treated mice before visible tumors were detected, suggesting that it was an early carcinogenic event (Fig. 5A).

Suppression of CCRK abrogates the circuitry to prevent hepatocarcinogenesis

To investigate whether perturbation of the CCRK-EZH2 circuitry affects HCC development, we tested if lentiviral-mediated silencing of *CCRK* can suppress tumor growth in the DEN-induced HCC model. Knockdown of CCRK significantly decreased the liver to body weight (p <0.05; Fig. 5B) and more importantly >70% tumor numbers in DEN-treated mice (p <0.01; Fig. 5C). Both tumor volume and weight in CCRK-silencing livers were also significantly decreased by >4-fold when compared to control livers (p <0.05 and p <0.01; Fig. 5D). Immunohistochemical staining of Ki67-positive tumor cells in CCRK-silencing livers was 3-fold less than that in control livers (p <0.001; Fig. 5E), indicating inhibition of aberrant hepatocellular proliferation by CCRK knockdown. The reduced tumorigenicity in DEN-treated mice was accompanied by suppression of the AR, p-AR^{Ser81}, CCRK, p-GSK3β^{Ser9}, active β-catenin, E2F1, p-AKT^{Ser473}, p-EZH2^{Ser21}, EZH2, and H3K27me3 levels (Fig. 5F, top) as well as transcriptional repression and

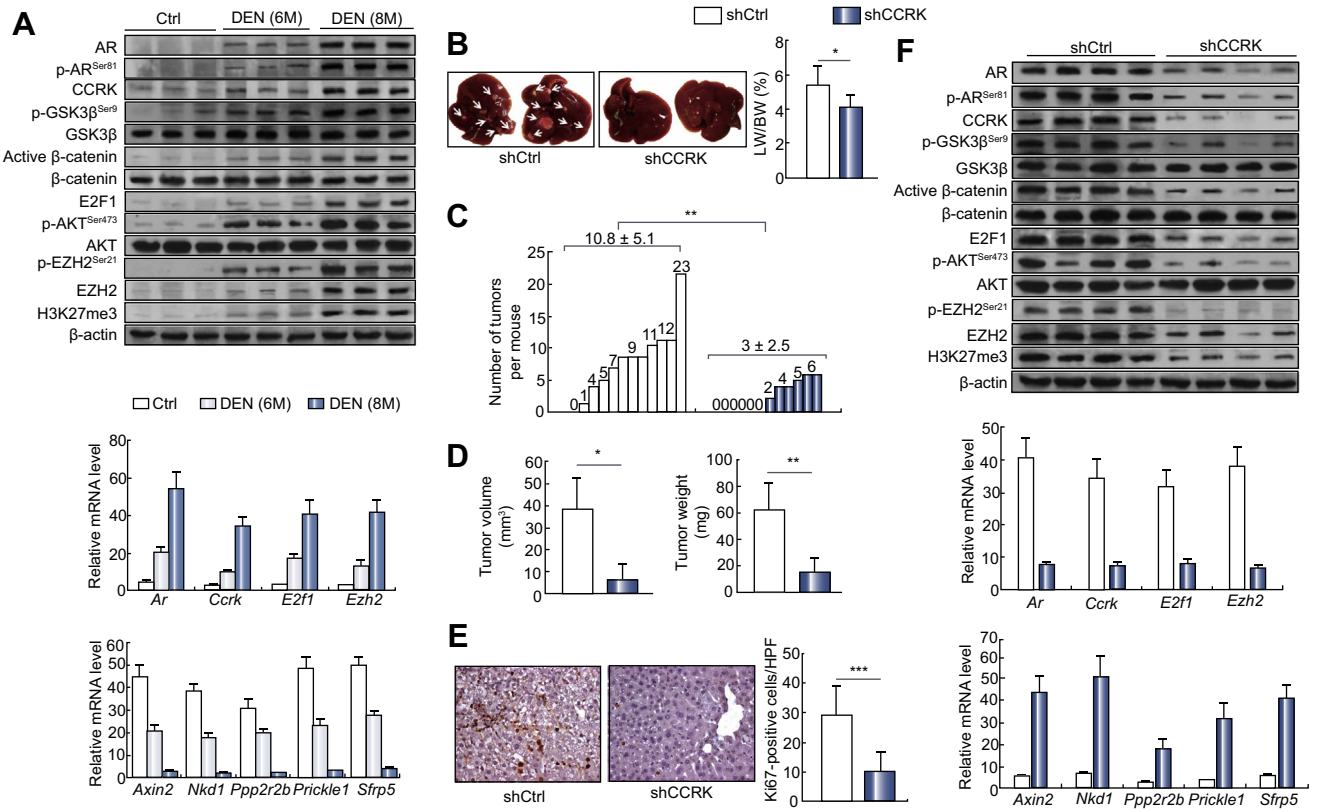

Fig. 5. Suppression of the CCRK-EZH2 circuit prevents HCC development in mice. (A) Immunoblot analysis of the CCRK-EZH2 circuit components (top) and quantitative RT-PCR analysis of the oncogenic factors (middle) and the Wnt antagonists (bottom) in untreated normal controls (Ctrl), and DEN-treated precancerous liver (DEN 6M) and HCC tissues (DEN 8M) from 6- to 8-month-old mice, respectively. (B) The liver to body weight, (C) numbers of tumors per mouse, (D) average tumor volume and weight and (E) average number of Ki67-positive proliferative cells per high-power field (HPF) in shCtrl-treated and shCCRK-treated groups were determined (n = 12 per group). Representative images of livers with tumor nodules indicated by *arrows* and Ki67 immunohistochemistry (×200 magnification) in both groups are shown. (F) Immunoblot analysis of the CCRK-EZH2 circuit components (top) and quantitative RT-PCR analysis of the oncogenic factors (middle) and the Wnt antagonists (bottom) in the liver tissues of the shCtrl-treated and shCCRK-treated groups. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. (This figure appears in color on the web.)

upregulation of the oncogenic factors and Wnt antagonists, respectively (p <0.001; Fig. 5F, bottom). Taken together, these data demonstrate that CCRK-EZH2 circuit activation is essential for HCC development *in vivo*.

The CCRK-EZH2 circuit components are concordantly over-expressed in human HCC tissues

To investigate the clinical relevance of our findings, the protein levels of AR, p-AR^{Ser81}, CCRK, p-GSK3β^{Ser9}, active β-catenin, E2F1, p-AKT^{Ser473}, p-EZH2^{Ser21}, EZH2, and H3K27me3 were examined in 34 pairs of human HCC tumor/non-tumor and 5 normal liver tissues. In contrast to the low basal levels in normal liver controls, marked upregulation of all protein expressions were observed in HCC tissues (Fig. 6A). Their average fold-changes of expressions in tumor tissues were also significantly higher than those of the paired non-tumor tissues (p <0.001; Fig. 6B). In the same cohort of samples, we examined the correlations between the expression levels among different circuit components. Notably, positive correlations between; (1) AR and p-AR^{Ser81}, (2) p-AR^{Ser81} and CCRK, (3) CCRK and p-GSK3β^{Ser9}, (4) p-GSK3β^{Ser9} and active β-catenin, (5) active β-catenin and E2F1, (6) E2F1 and EZH2, (7) EZH2 and H3K27me3, (8) active β-catenin and AR, (9) CCRK and p-AKT^{Ser473} as well as (10) p-AKT^{Ser473}, and p-EZH2^{Ser21} revealed a corresponding over-expression in human HCCs (Pearson's correlation coefficients: r = 0.785–0.906; p <0.001; Fig. 6C–D). Moreover, stepwise increases in protein expressions were observed along hepatocarcinogenesis, namely normal livers to adjacent precancerous liver tissues to HCC tumors (Fig. 6C). Interestingly, we found that the circuit components exhibited higher expressions in both male precancerous liver and HCC tissues compared to the female counterparts as shown in the Western blots (Fig. 6A) and the correlation graphs (Fig. 6C).

Hyperactivation of the CCRK-EZH2 circuitry correlates with poor prognosis of patients

We investigated if the CCRK-EZH2 circuitry is perturbed during HCC progression. We found that the protein expression levels of all the circuit components were increased from early to intermediate/advanced stages of HCC (p <0.05; Fig. 7A). Moreover, their expressions were higher in poorly-differentiated than moderately- or well-differentiated HCCs (p <0.05 to 0.01; Fig. 7B). Finally, we determined whether the activity of the CCRK-EZH2 circuitry associates with HCC prognosis. The activity was determined by the averaged fold-change of all circuit component expressions in tumor *vs.* non-tumor tissues (Supplementary Table 1). Hyperactivation of the CCRK-EZH2 circuitry was significantly associated with reduced serum albumin level, poor

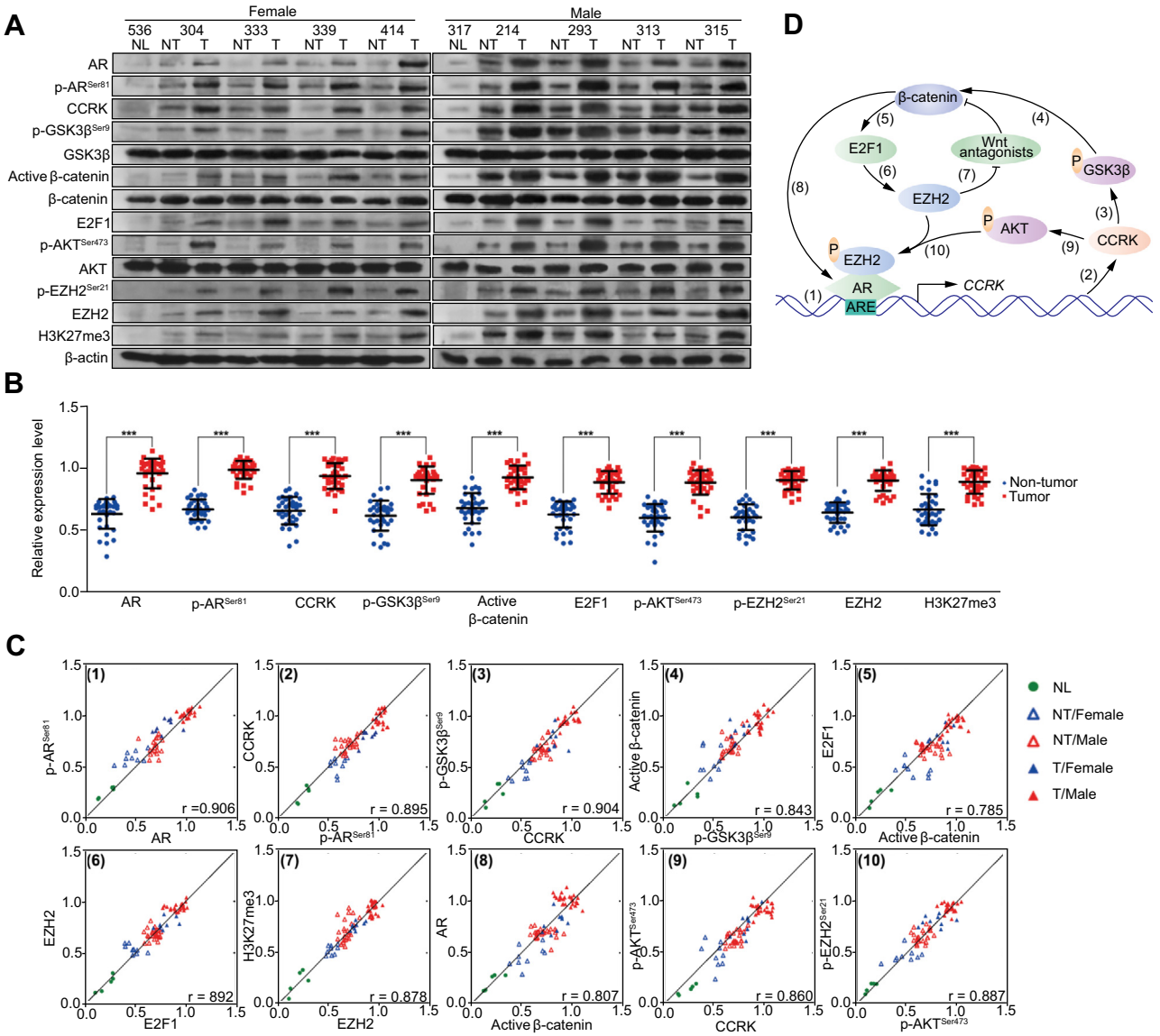

Fig. 6. The CCRK-EZH2 circuitry is activated in human HCC tissues. (A) Immunoblot analysis of the CCRK-EZH2 circuit components in human tissues. Representative blots from normal liver (NL), adjacent non-tumor (NT) and tumor (T) tissues of both sexes are shown. (B) Relative protein expression levels of 34 paired HCC tumor and NT tissues. The expression levels of p-GSK3β^{Ser9}, active β-catenin and p-AKT^{Ser473} were normalized by total GSK3β, β-catenin and AKT, respectively. The remaining protein expression levels were normalized by β-actin. The lines in the dot plot denote mean ± standard deviation. ***p <0.001. (C) Correlation of the expression levels among different CCRK-EZH2 circuit components in 5 NLs (*green*), 25 male (*red*) and 9 female (*blue*) paired HCC tumor (*closed triangle*) and NT (*open triangle*) tissues denoted with Pearson's correlation coefficients. (D) Schematic representation of the CCRK-EZH2 epigenetic circuitry in hepatocarcinogenesis, with the sequence of the regulatory interactions numbered as the correlation plots in *C*.

differentiation and recurrence of HCC (p <0.05; Supplementary Table 2). Most strikingly, Kaplan-Meier analysis showed that HCC patients with high circuit activity were significantly associated with shorter overall (hazard ratio = 20.99, p <0.0001; Fig. 7C) and disease-free survival rates (hazard ratio = 6.04, p <0.01; Fig. 7D).

Discussion

Accumulating evidence has unmasked the molecular linkage between sex hormone signaling and gender disparity in HCC incidence [25–27]. Although aberrant chromatin modifications and remodeling have emerged as hallmarks of the male-predominant HCC [4,6,13], whether mechanistic links exist between chromatin regulator and AR signaling have yet to be delineated. Our data reveal a tumor-initiating circuitry constituted by the reciprocal regulation of CCRK and EZH2. The dual kinase activities of CCRK regulate the transcription and post-translation of EZH2. The histone methyltransferase and co-activator functions of EZH2 in turn upregulate AR expression and transcriptional activity, respectively, to trans-activate *CCRK*, thus establishing an epigenetic vicious cycle (Fig. 6D). Epigenetic switches mediated by microRNA transcription factor regulatory loops play critical roles in HCC initiation [28,29]. In the context of AR-induced hepatocarcinogenesis, we propose a

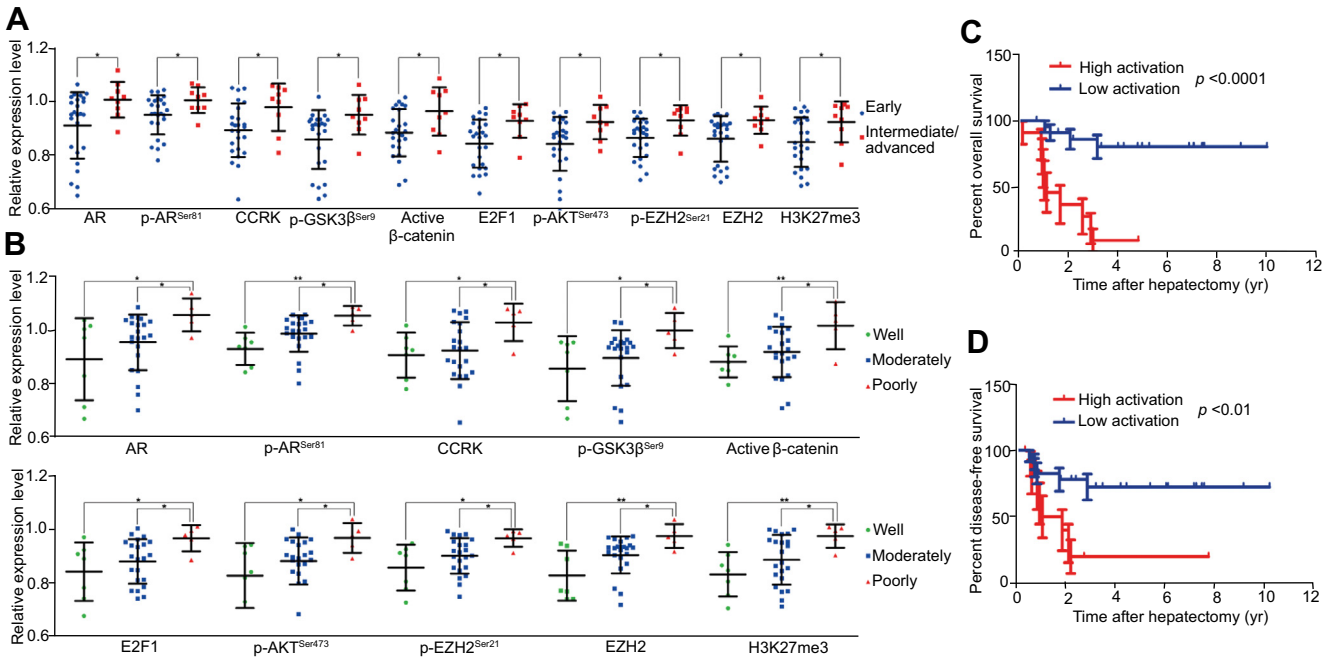

Fig. 7. Hyperactivation of the CCRK-EZH2 circuitry correlates with short survival of patients. (A) Relative protein expression levels of the CCRK-EZH2 circuit components in 34 HCC tumor tissues stratified by tumor stage and (B) differentiation. $^{*}p$ <0.05; $^{**}p$ <0.01. The lines in the dot plot denote mean ± standard deviation. (C) Kaplan-Meier overall survival and (D) disease-free survival curves of HCC patients with high (n = 11) and low (n = 23) activity of the CCRK-EZH2 circuitry.

model of epigenetic circuitry in which transient androgen/AR signaling is converted to a continuous signal by EZH2 activities. Downregulation of EZH2 abolishes the pro-proliferative and malignant transformation functions of CCRK, mechanistically linking epigenetic reprogramming and liver cancer. The gender difference expression pattern of the CCRK-EZH2 circuit members in human HCCs further corroborates the biological significance of this self-reinforcing circuitry in male hepatocarcinogenesis.

It is becoming increasingly clear that oncogenic proteins interact through feedback and feed-forward loops in complex signaling networks [30]. Our data suggests that CCRK functions as a signaling hub to connect multiple kinases (GSK-3β and AKT) and transcriptional regulators (AR, β-catenin, TCF, E2F1, EZH2) which play critical roles in HCC pathogenesis [2,3,7]. Intriguingly, a positive feedback loop is identified within this phosphorylation-transcriptional network, whereby a GSK-3β/β-catenin/TCF/E2F1 cascade trans-activates *EZH2*, which binds to and represses the promoters of Wnt antagonists via PRC2-mediated H3K27me3, leading to increased β-catenin/TCF and E2F1 transcriptional activities. Such 'wheel within a wheel' mechanism provides additional epigenetic input into β-catenin/TCF-mediated transcription to maintain preferentially high AR expression in HCC.

In addition to the transcriptional feedback loop, we also identified a feed-forward loop in which CCRK induces EZH2 phosphorylation, thereby promoting p-EZH2^{Ser21}-AR physical interaction for *CCRK* promoter co-occupancy and transcriptional activation. Contrary to the H3K27me3-mediated repression of the Wnt antagonists, phosphorylated EZH2 functions as an AR co-activator to establish an active chromatin state at the *CCRK* promoter that is accessible to RNA polymerase II. While the SET domain is required for epigenetic activation of β-catenin signaling, serine21 phosphorylation of EZH2 may also contribute to AR induction via facilitating complex formation and promoter binding with β-catenin (data not shown). Our findings suggest that AKT is a new oncogenic mediator of CCRK and strengthens the notion that AKT-mediated EZH2 phosphorylation at serine21 is a critical modification for its PRC2-independent functions in prostate and brain cancers [22,23,31]. While CCRK has also been shown to promote the male-predominant glioblastoma [32], the interplay between CCRK and EZH2 pathways in this cancer warrants further investigation. Taken together, our findings suggest that CCRK-activated GSK-3β and AKT phosphorylation endorse EZH2 targeting of both histone and non-histone substrates, respectively, to contribute to the transcriptional program in HCC.

As hundreds of millions of people worldwide are still at a high risk of new HCC development due to viral infection and obesity, the definition of molecular aberrations suitable for prevention is especially important in this malignancy. Our recent finding of CCRK mediating the viral-host interaction in HBV-associated HCC [19] suggests its involvement in the early carcinogenic process. We found that the HBV X oncoprotein augments a CCRK/GSK-3β/β-catenin/AR regulatory loop in which its feedback upregulates its own expression to promote hepatocellular proliferation and transformation [19]. In this study, we further delineated an essential epigenetic component of the molecular circuitry and showed that activation of this vicious cycle precedes the formation of tumors in DEN-treated mice. More importantly, expression analysis of the clinical specimens revealed significant stepwise increases of the CCRK-EZH2 circuit components along the carcinogenic cascade. Using a similar DEN-treated model and a cohort of patient samples, He *et al.* have recently identified HCC progenitor cells in the premalignant lesions [33]. Autocrine interleukin-6 signaling, which constitutes the inflammatory feedback circuits [28,29], plays a pivotal role for the transformation of these HCC progenitors [33]. Delineating the potential molecular connection between the CCRK-EZH2 and inflammatory circuits

may provide further insights into HCC initiation and prevention strategies.

Our clinical data demonstrate that the CCRK-EZH2 circuitry is important not only for HCC initiation but also progression. In a relatively small cohort of human HCCs, over-expression of the CCRK-EZH2 circuit members is observed, which significantly correlates with tumor recurrence and poor survival of patients. The rare commonality of actionable mutations between individual HCC cases raises the possibility that alteration of 'epi-driver genes' may be an alternative 'Achilles heel' in liver cancer [10,11]. Silencing of CCRK dephosphorylates the kinases, suppresses the transcriptional regulators and perturbs their downstream targets *in vivo*, thus supporting its central role in regulating the phosphorylation-transcriptional network. The dramatic inhibitory effects of CCRK knockdown on hepatic tumorigenicity further provide proof-of-principle for the development of selective small-molecule kinase inhibitors [34]. In conclusion, this study identifies a novel molecular circuitry that drives malignant progression and delineates the mechanistic details of EZH2 bi-functionality as both transcriptional repressor and co-activator in HCC. Exploiting these epigenetic vulnerabilities via the CCRK signaling hub may offer unique potential for HCC prevention and treatment.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Author's contributions

Study concept and design: J.J.Y.S., A.S.L.C.; acquisition of data: H.F., Z.Y., Y.T., Y.-Y.L., M.S.L.; analysis and interpretation of data: H.F., Y.T., A.S.L.C.; drafting of the manuscript: H.F., A.S.L.C.; critical revision of the manuscript for important intellectual content: P.B.S.L., A.M.L.C., K.-F.T., H.L.Y.C., J.J.Y.S., A.S.L.C.; statistical analysis: F.H., Z.Y.; obtained funding: H.L.Y.C., J.J.Y.S., A.S.L.C.; administrative, technical, or material support: Y.-S.C., P.B.S.L., A.M.L.C., K.-F.T.; study supervision: J.J.Y.S., A.S.L.C.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.11.040.

References

[1] El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology 2012;142:e1261.
[2] Tian Y, Wong VW, Chan HL, Cheng AS. Epigenetic regulation of hepatocellular carcinoma in non-alcoholic fatty liver disease. Semin Cancer Biol 2013;23:471–482.
[3] Villanueva A, Llovet JM. Targeted therapies for hepatocellular carcinoma. Gastroenterology 2011;140:1410–1426.
[4] Villanueva A, Llovet JM. Liver cancer in 2013: mutational landscape of HCC–the end of the beginning. Nat Rev Clin Oncol 2014;11:73–74.
[5] Han ZG. Functional genomic studies: insights into the pathogenesis of liver cancer. Annu Rev Genomics Hum Genet 2012;13:171–205.
[6] Yu Z, Cheng AS. Epigenetic deregulation of microRNAs: new opportunities to target oncogenic signaling pathways in hepatocellular carcinoma. Curr Pharm Des 2013;19:1192–1200.
[7] Zender L, Villanueva A, Tovar V, Sia D, Chiang DY, Llovet JM. Cancer gene discovery in hepatocellular carcinoma. J Hepatol 2010;52:921–929.
[8] Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res 2009;69:7385–7392.
[9] Stauffer JK, Scarzello AJ, Andersen JB, De Kluyver RL, Back TC, Weiss JM, et al. Coactivation of AKT and beta-catenin in mice rapidly induces formation of lipogenic liver tumors. Cancer Res 2011;71:2718–2727.
[10] Knudsen ES, Gopal P, Singal AG. The changing landscape of hepatocellular carcinoma: etiology, genetics, and therapy. Am J Pathol 2014;184:574–583.
[11] Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz Jr LA, Kinzler KW. Cancer genome landscapes. Science 2013;339:1546–1558.
[12] You JS, Jones PA. Cancer genetics and epigenetics: two sides of the same coin? Cancer Cell 2012;22:9–20.
[13] Zhang Z. Genomic landscape of liver cancer. Nat Genet 2012;44:1075–1077.
[14] Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. Nature 2011;469:343–349.
[15] Tsang DP, Cheng AS. Epigenetic regulation of signaling pathways in cancer: role of the histone methyltransferase EZH2. J Gastroenterol Hepatol 2011;26:19–27.
[16] Suva ML, Riggi N, Bernstein BE. Epigenetic reprogramming in cancer. Science 2013;339:1567–1570.
[17] Ma WL, Lai HC, Yeh S, Cai X, Chang C. Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis. Endocr Relat Cancer 2014;21:R165–R182.
[18] Feng H, Cheng AS, Tsang DP, Li MS, Go MY, Cheung YS, et al. Cell cycle-related kinase is a direct androgen receptor-regulated gene that drives beta-catenin/T cell factor-dependent hepatocarcinogenesis. J Clin Invest 2011;121:3159–3175.
[19] Yu Z, Gao YQ, Feng H, Lee YY, Li MS, Tian Y, et al. Cell cycle-related kinase mediates viral-host signalling to promote hepatitis B virus-associated hepatocarcinogenesis. Gut 2014;63:1793–1804.
[20] Cheng AS, Lau SS, Chen Y, Kondo Y, Li MS, Feng H, et al. EZH2-mediated concordant repression of Wnt antagonists promotes beta-catenin-dependent hepatocarcinogenesis. Cancer Res 2011;71:4028–4039.
[21] Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 2003;22:5323–5335.
[22] Kim E, Kim M, Woo DH, Shin Y, Shin J, Chang N, et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. Cancer Cell 2013;23:839–852.
[23] Xu K, Wu ZJ, Groner AC, He HH, Cai C, Lis RT, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. Science 2012;338:1465–1469.
[24] Lee JS, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, et al. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nat Genet 2004;36:1306–1311.
[25] Awuah PK, Monga SP. Cell cycle-related kinase links androgen receptor and beta-catenin signaling in hepatocellular carcinoma: why are men at a loss? Hepatology 2012;55:970–973.

[26] Li Z, Tuteja G, Schug J, Kaestner KH. Foxa1 and Foxa2 are essential for sexual dimorphism in liver cancer. Cell 2012;148:72–83.
[27] Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317:121–124.
[28] Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, et al. An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell 2011;147:1233–1247.
[29] Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell 2009;139:693–706.
[30] Yap TA, Omlin A, de Bono JS. Development of therapeutic combinations targeting major cancer signaling pathways. J Clin Oncol 2013;31:1592–1605.
[31] Cha TL, Zhou BP, Xia W, Wu Y, Yang CC, Chen CT, et al. Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. Science 2005;310:306–310.
[32] Ng SS, Cheung YT, An XM, Chen YC, Li M, Li GH, et al. Cell cycle-related kinase: a novel candidate oncogene in human glioblastoma. J Natl Cancer Inst 2007;99:936–948.
[33] He G, Dhar D, Nakagawa H, Font-Burgada J, Ogata H, Jiang Y, et al. Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. Cell 2013;155:384–396.
[34] Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer 2009;9:28–39.